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TITLE: Characterization of Prostate-Specific Membrane Antigen (PSMA) for Use in Therapeutic and Diagnostic Strategies Against Prostate Cancer

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13. ABSTRACT *(Maximum 200 Words)*

Prostate-Specific Membrane Antigen (PSMA) appears to be an ideal prostate cancer marker and potential therapeutic target, however there have been reports of PSMA expression in non-prostatic tissues, including brain, kidney and liver. Such expression of PSMA could weaken the potential of the gene as a prostate cancer marker or at least produce confusing and conflicting data. Prior to this project, we had demonstrated that there was another human gene, 97% identical to PSMA, which we termed the PSMA-Like gene. The first aim of this project was to characterize the differences between the non-prostatic and prostatic forms of PSMA and to determine strategies to specifically target PSMA expressed in prostate or prostate tumors. The second aim of the project was to define the minimal regulatory regions of the PSMA gene, for future utilization in gene therapy strategies, by analyzing deletion constructs of the gene regulatory regions. To date we have cloned and characterized the PSMA-Like gene, and determined ways of distinguishing the two genes at the DNA, RNA and protein levels which will aid in designing new diagnostic and therapeutic anti-prostate cancer strategies. In addition, we have characterized the promoter and enhancer region of PSMA, and developed gene therapy vectors that specifically kill human prostate cancer tumors growing in mice.

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Introduction

Prostate-Specific Membrane Antigen (PSMA) appears to be an ideal prostate cancer marker and potential therapeutic target, however there have been reports of PSMA expression in non-prostatic tissues, including brain, kidney and liver. Such expression of PSMA could weaken the potential of the gene as a prostate cancer marker or at least produce confusing and conflicting data. Prior to the onset of this project, we had demonstrated that there was another human gene, approximately 97% identical to PSMA, which we termed the PSMA-Like gene. Therefore the first aim of this project was to characterize the differences between the non-prostatic and prostatic forms of PSMA at the nucleic acid, protein, and functional levels and to determine strategies to specifically target PSMA expressed in prostate or prostate tumors. The second aim of the project was to define the minimal regulatory regions of the PSMA gene, for future utilization in gene therapy strategies, by analyzing deletion constructs of the gene regulatory regions and by comparison with the PSMA-Like gene regulatory regions.

Specific Aim 1. *To clone and sequence the "PSMA-Like" gene, and comparatively analyze tissue expression and function of the PSMA and PSMA-Like genes.*

As described in my first two reports, I have cloned and characterized the tissue distribution and enzymatic activity of the PSMA-Like gene (now designated Folh1B by Genbank; accession number AF261715). The genomic sequence of the PSMA and PSMA-Like genes is 97% identical, however the PSMA-Like gene has undergone a deletion of the region corresponding to the PSMA promoter, exon one and part of intron one. Therefore, the PSMA-Like gene uses an alternative promoter which is found in intron five of the gene. Interestingly, when I attempted to develop RNase protection assays and RT-PCRs assuming that the transcribed region of intron five in PSMA-Like would differentiate it from PSMA, I found that in fact PSMA also initiates transcription from this promoter, although not at very high levels. I was able to distinguish the two genes using RT-PCR followed by restriction enzyme digestion and using this method determined that the PSMA-Like gene is expressed in kidney and liver. The PSMA gene is expressed in prostate, prostate cancer, tumor neovasculature, the brain and small intestine, and in addition we see PSMA RNA in kidney and liver. The cDNA sequence of the PSMA-Like gene is 1992 bp, and translation predicts a protein of 443 amino acids or 49kD in size, as well as a cytosolic location in the cell.

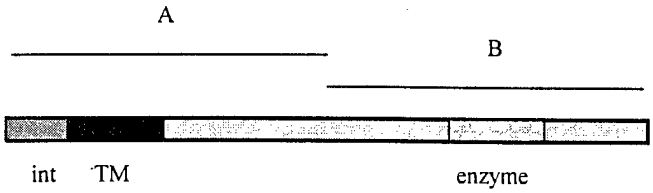
In the final six months of the project, I further analysed in the expression pattern of PSMA and PSMA-Like, as described in the accompanying paper (O'Keefe et al., submitted). We were able to design a probe based on the genomic DNA region deleted in the PSMA-Like gene and use it to specifically determine where PSMA is expressed via northern analysis. In addition, we quantified the expression of PSMA in various tissues relative to expression in the normal prostate. Significant levels of PSMA are expressed in all regions of the human brain that we examined. In addition, the highest level of PSMA expression in the body seems to be in the spinal cord, followed by prostate. The next highest-expressing tissue was kidney, which was less than 10% of the amount seen in the normal prostate. We were able to detect expression of both the PSMA and PSMA-Like genes simultaneously by using the entire PSMA-Like coding

sequence as a probe. The PSMA-Like gene was only expressed in kidney and liver, at approximately equivalent levels to PSMA. Interestingly, the PSMA gene is expressed in fetal kidney and liver, and adult trachea – all of which are previously unreported observations. To confirm that PSMA protein was expressed, I performed western blot analyses. I used an antibody (anti-PSMA PM2M 440, kindly supplied by Hybritech Inc., San Diego, CA), that will only detect PSMA and not PSMA-Like protein. I was able to clearly show expression of PSMA protein in the brain, specifically in hippocampus and amygdala (the only tissues I could obtain). As PSMA is currently being used as a target for cytotoxic therapies, I would recommend that any patients be closely monitored for liver and kidney damage. The brain is protected by the blood brain barrier, and as the current therapies are antibody based, they will not cross into the brain which should therefore be protected.

Functional Analysis of the PSMA-Like gene

These studies have been somewhat difficult. Last year, I reported that we had expressed the PSMA-Like gene in a cell line and shown the cell line gained NAALADase activity, one of the enzymatic activities of PSMA. However this data was based on one experiment, which I attempted to repeat a significant number of times before publishing. I was unable to repeat this result. In addition, I was unable to establish stable cell lines expressing PSMA-Like, although I repeatedly had no problem establishing stable cell lines with my control experiments (vector alone and vector containing PSMA). I next optimized transient transfections of PC3 cells so as NAALADase activity could be examined after transfection of a PSMA containing vector. Repeatedly I was unable to demonstrate PSMA-Like enzymatic activity. Western blot analysis with antibodies against PSMA demonstrated PSMA protein present after transient transfection, but was not able to show PSMA-Like protein. This could be because antibodies such as Cyt-351 bind to the N-terminus of PSMA or other regions that are missing in PSMA-Like. Because the amino-acid differences between the two proteins in the presumed enzymatic region are so few, it was possible that folding of the PSMA-Like protein was affected by the lack of a transmembrane domain, leading to loss of enzymatic activity. I next created a "hybrid" molecule, containing the first 307 amino acids of PSMA, followed by all of PSMA-Like (see figure 1).

Figure 1: Diagrammatic representation of the PSMA/PSMA-Like hybrid molecule. The Cyt-351 antibody binds the internal domain (int) of PSMA. TM refers to the transmembrane domain, enzyme to the enzymatic pocket. A indicates the first 307 amino acids of PSMA, B indicates the entire protein sequence of PSMA-Like, together they form the hybrid.



Following transient transfection into PC-3 cells, I was able to demonstrate appropriate expression of the PSMA/PSMA-Like hybrid molecule via western analysis using the Cyt-351 antibody which binds the amino-terminal of PSMA. Surprisingly, this construct did not have NAALADase activity. In the last six months of the project, we were able to develop an HPLC based method for examining the folate hydrolase activity of PSMA. The chimeric construct also

had no folate hydrolase activity. This is surprising as PSMA and the PSMA/PSMA-Like only differ by nine amino-acids. Of these, six are similar amino acids. In addition, these six amino acids that differ are all found in at least one of the other species for which a NAALADase homolog has been cloned (e.g. rat, mouse, pig), that do exhibit NAALADase activity and therefore we do not think these changes are responsible for the loss in activity. Of the three other differences, two are found near the zinc-ligand binding regions of the enzyme, and also the glutamic acid that is thought to be necessary for catalysis. These changes are:

a.a. 398 isoleucine→threonine (PSMA→PSMA-Like)
a.a. 437 glutamic acid→aspartic acid (PSMA→PSMA-Like)

Site-directed mutagenesis of amino acids close to these residues has previously been shown to reduce the enzyme's activity. Therefore these residues might be a good target for small molecule based drugs.

Specific Aim Two. *To define the minimal promoter/enhancer regions of the PSMA gene and determine the specific sequence elements responsible for activity.*
In my last two reports I stated that the DNA sequence in the PSMA-Like gene that corresponds to the promoter, exon one and part of intron one of the PSMA gene, has been deleted. As such, PSMA-Like uses an alternative promoter found in the region corresponding to intron five of the gene. This promoter is entirely different to the PSMA promoter. The enhancer regions of the two genes however, are **99.5%** identical (I have deposited the sequence of the PSMA-Like enhancer region into Genbank, accession number AF480875). If the PSMA-Like enhancer was not able to drive activity in prostate cells, we could use the differences to determine the critical regions for activity in the PSMA enhancer. However, we found that in combination with the PSMA promoter, the PSMA-Like enhancer was in fact able to drive prostate-specific reporter gene activity, with over 80% of the activity of the PSMA enhancer. However, in collaboration with Dr. Peter Molloy's laboratory in Australia, we were able to determine that the PSMA-enhancer is able to drive prostate-specific expression using heterologous promoters, including the PSA and probasin promoters which are prostate derived promoters, and the non-tissue specific herpes virus TK promoter. In addition, the most active portion of the PSMA enhancer is a 331bp region, which, like the native PSMA gene, is upregulated by androgen deprivation. In our aim to design a gene therapy approach against prostate cancer, utilizing the PSMA enhancer, I last reported that we had generated such a vector. The vector drives the *E.coli* Cytosine deaminase gene and we were able to demonstrate cytotoxicity of this vector in prostate cancer but not control cell lines treated with the non-toxic prodrug 5-fluorocytosine (5-FC). In collaboration with Dr. Atsushi Uchida, a clinical fellow whom I have been assisting in his research training in the lab, we were able to show that treatment with this vector and 5-FC in nude mice with LNCaP-C42 prostate cancer cell xenografts resulted in significantly smaller, necrotic and non-hemorrhagic tumors in the treatment group (refer to figures and data provided with previous report).

In the final six months of this project, I assisted a Urology resident in his research year. We attempted to improve our previous "gene therapy" results by enhancing the activity of the cytosine deaminase "suicide" gene. We hypothesised that if we expressed cytosine deaminase

protein outside the cell, we would see a greater bystander affect than seen with cytosolic cytosine deaminase. To accomplish this, we produced a chimeric protein containing the transmembrane domain and cytosolic domain of the PSMA gene, followed by the entirety of the yeast cytosine deaminase gene. We used the PSMA transmembrane domain and internal domain because we have an antibody which allows us to detect it. In addition, we chose the yeast cytosine deaminase gene as opposed to the *E. coli* gene we previously used, as this had recently been reported to be several-fold more active than the bacterial gene. Also, the yeast gene is 474 bp long, compared to more than 1000 bp for the bacterial gene. This is important, because we finally intend to utilize this construct in a gene therapy vector that most-likely has constraints on how much foreign DNA it can carry. Stable cell lines were made expressing either yeast cytosine deaminase (yCD) or the chimeric protein (TMyCD) under control of the PSMA promoter and enhancer on the C4-2 cell line background. Experiments examining the efficiency of prodrug conversion revealed that the TMyCD line had 10 fold activity over the yCD lines, but only about the same amount of activity as our original cell lines transfected with the bacterial CD gene. It was unclear if this was due to the amount of protein being expressed or some other factor, and this project is currently being pursued by a clinical resident.

The final part of aim two was to isolate the specific sequences responsible for activation of the PSMA enhancer. As I mentioned previously, this was especially difficult as the PSMA-enhancer has specific regions that both up-regulate and down-regulate expression. However, during the final six months of the project, I was testing the enhancer construct in a new cell line in the lab, MDAPCa2b. This cell line expresses PSMA at ten fold higher levels than the C4-2 or LNCaP cell lines. Interestingly, both the PSMA and PSMA-Like enhancers in combination with the PSMA promoter drive reporter gene activity in the MDAPCa2b cell line, and although both enhancers work equally well in C4-2 cells, the PSMA enhancer works more than 2.5 fold as well in the MDAPCa2b cell line than the PSMA-Like enhancer does (see figure 2 below; see attached manuscript O'Keefe et al. 2002, submitted).

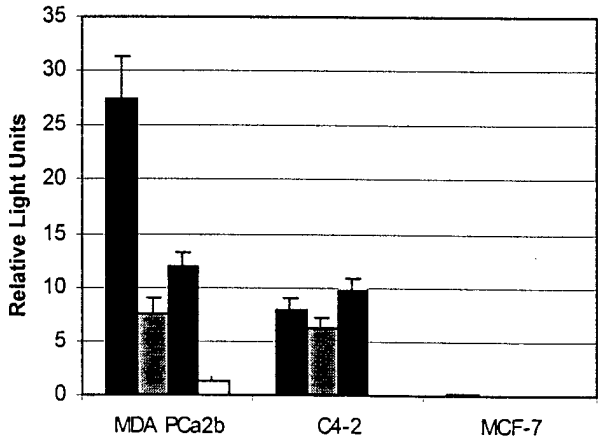


Figure 2: Reporter gene expression in MDAPCa2b, C4-2 and MCF-7 cells. Solid boxes indicate the PSMA enhancer activating the PSMA promoter, wavy boxes are the PSMA-Like enhancer activating the PSMA promoter, while the clear box indicates PSMA promoter alone.

As there are so few differences between the two enhancers, we can now start to isolate the regions responsible for this difference in activity and determine what factor activates them, and these data will provide the basis for another grant.

Key Research Accomplishments: July 1, 2001 – December 31, 2001

- I have demonstrated and quantitated via northern analysis significant levels of PSMA mRNA in a number of human tissues; including every region of the brain examined, trachea, kidney and liver. Suprisingly, I found that the highest PSMA expressing tissue in the body is the spinal cord, followed by normal prostate. In addition PSMA is expressed in fetal liver and kidney.
- I have examined a large number of human tissues for expression of the PSMA-Like gene, and demonstrated that it is expressed in adult kidney and liver, at about the same level the PSMA gene is.
- I have demonstrated PSMA protein expression in the brain; using hippocampus and amygdala as representative tissues. These data and those described above obviate the need to take care when designing therapeutic strategies targeting PSMA.
- I have comparatively analyzed the 1.6kb enhancer regions of both the PSMA and PSMA-Like genes, that differ by a few base pairs. I was able to show that while both enhancers regulate reporter gene expression equally well in the C4-2 cell line, the PSMA enhancer is upregulated more than 2.5 fold over the PSMA-Like enhancer when transfected into the MDAPCa2b cell line. This indicates that one or more of the few base pair differences between the two enhancer sequences corresponds to an enhancer binding factor that strongly increases expression and is found in the MDAPCa2b cell line, but not the C4-2 cell line.
- Finally, together with a Urology Resident, Dr. Aaron Milbank, I have designed and tested a novel suicide gene for use in PSMA-enhancer driven therapeutic constructs. The gene produces a chimeric protein consisting of the first part of PSMA, including the recognition site for the clinical imaging agent, Prostascint, followed by the transmembrane domain of PSMA attached via a linker region to the yeast cytosine deaminase gene. Initially testing of this construct versus yeast cytosine deaminase alone has given some encouraging results, and if viable for human gene therapy would able to be visualized in the patient.

Reportable Outcomes: July 1, 2001 – December 31, 2001

Manuscripts / Book Chapters

1. Uchida, A., **O'Keefe, D.S.**, Bacich, D.J., Molloy, P.L. and Heston, W.D.W. *In vivo* suicide gene therapy model using a newly discovered Prostate-Specific Membrane Antigen (PSMA) Promoter/Enhancer: A potential alternative approach to androgen deprivation therapy. (2001). Urology 58:132-139
2. Bacich, DJ, Ramadam, E, **O'Keefe, D.S.,** Bukhari, N, Wegorzewska, E, Ojeifo, O, Wrenn, CC, Bzdega, T, Wroblewska, B, Heston, WDW, Neale JH. Deletion of the Glutamate Carboxypeptidase II Gene in Mice reveals a second enzyme activity that hydrolyzes N-Acetylaspartylglutamate. *(J. Neurochem, in press.)*

3. Balaji, K.C., Louis, S., Rao, P.S., Tiourin, M., Sherman, S., Hock, L.M., Bacich D.J. and **O'Keefe, D.S.** Microarray analysis of differential gene expression in androgen independent prostate cancer using a metastatic human prostate cancer cell line model *(submitted)*.
4. **O'Keefe, D.S.,** Bacich, D.J. and Heston, W.D.W. Comparative Analysis of Prostate-Specific Membrane Antigen (PSMA) versus a Prostate-Specific Membrane Antigen-*Like* Gene (*submitted*).

Abstracts/Presentations

5. Bacich, D.J., **O'Keefe, D.S.**, Heston, W.D.W, Callizot, N., Poindron, P., Tiffany, C., Wozniak, K., Slusher, B. Glutamate Carboxypeptidase II (NAALADase) Knockout Mice are Less Susceptible to Chemical and Injury-Induced Neuropathy. Abstract 259.19, Society for Neuroscience 31st Annual Meeting, San Diego, CA, November 2001.
6. Bzdega, T., **O'Keefe, D.S.**, Bacich, D.J., Ramadan, E., Wroblewska, B., Bukhari, N., Wegorzewska, I., Heston, W.D.W, Neale, J.H. Generation and Characterization of mice lacking Glutamate Carboxypeptidase II. Abstract 477.9, Society for Neuroscience 31st Annual Meeting, San Diego, CA, November 2001.
7. **O'Keefe, D.S.,** Uchida, A., Bacich, D.J., Milbank, A. and Heston, W.D.W. An In vivo Suicide Gene Therapy Model using the Prostate-Specific Membrane Antigen Promoter/Enhancer. Abstract A-42, AACR Special Conference "New Discoveries in Prostate Cancer Biology and Treatment", December, 2001.
8. **O'Keefe, D.S. and Heston, W.D.W.** Comparative analysis of the PSMA and PSMA-Like genes yields some surprising insights. Abstract 1958, AACR National Conference, April 2002.

Other Training Accomplishments

For this six-month period I assisted in the training of a Urology Resident in his laboratory research year. We presented the results of the resident's project at the AACR special conference on Prostate Cancer in Naples, 2001, and the resident has presented his work at the AACR National Conference (2002) and also at AUA (2002). Following my career plan I am applying for tenure-track assistant professor positions at comprehensive cancer centers. My future research aims have however changed and I am currently writing a grant proposal that examines the role of PSMA in folate metabolism in prostate cancer/the normal prostate and what possible effects expression of PSMA and its isoforms might have on DNA damage and DNA/histone methylation in patients whose folate intake is insufficient.

Personnel receiving compensation from this award:

Denise S. O'Keefe

Conclusions

This project set out to determine the differences in sequence and function of the PSMA and PSMA-Like genes. PSMA is a very important clinical target for prostate cancer, and the presence of another highly similar gene needed to be investigated to maximize the utility of PSMA as a tumor marker and target. We have cloned and characterized both the PSMA-Like gene and it regulatory regions, and carried out a comparative analysis with the PSMA gene. We have shown that antibodies used for clinical imaging, such as the cyt351 antibody (Prostascint) will not cross-react with PSMA-Like protein. In addition, because PSMA-Like is not on the surface of the cell, it is not available for treatment methods designed to target PSMA. Clinical tests which were based on RT-PCR of PSMA from peripheral blood or urine could be

resurrected using oligonucleotide primers that will not detect the PSMA-Like gene. Previously, it was thought that PSMA transcripts were non-specifically found in cells isolated from blood and urine, with no clinical relevance. As PSMA-Like is expressed in the kidney, it is especially likely that cells expressing PSMA-Like could escape into the urine, possibly confounding these results. We have now established ways to differentiate the PSMA and PSMA-Like genes at the DNA, RNA and protein levels, and used these methods to confirm expression of PSMA in brain, kidney and liver both at the RNA and protein level. **Expression of PSMA protein at significant levels in the brain, kidney and liver is concerning, and patients being treated with therapies that are cytotoxic to cells expressing PSMA should be monitored carefully for other tissue damage.**

The second part of this project is essentially aimed at defining the regulatory regions of PSMA for use in future gene therapy constructs. We have defined the best region of the enhancer for prostate-specific expression, and generated a gene therapy vector that combined with a "suicide" gene and non-toxic prodrug therapy, this enhancer is able to kill both prostate cancer cells in culture, and in an (immunodeficient) mouse model, *in vivo*. Future studies are aimed at maximizing the suicide gene activity by genetically enhancing it, and also on delivery methods that could be used in humans to treat prostate cancer. In addition, by comparative analysis with the PSMA-Like enhancer, we have been able to localize to a number of specific sequences regions responsible for enhancer binding factors that significantly increase reporter gene expression in a prostate cancer cell line.

Appendix

Curriculum Vitae

PII Redacted

Name: **Denise Susan O'Keefe, PhD.**

[redacted]

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Education:

1987-1989: Bachelor of Science awarded by La Trobe University, Melbourne, Australia. Double major in Human Genetics and Microbiology.

1990: Honors degree in Genetics and Human Variation awarded by La Trobe University, Melbourne, Australia.

1991-1995: Doctorate of Philosophy, in the Department of Hematology-Oncology, the Faculty of Medicine, at The Queen Elizabeth Hospital, The University Of Adelaide, Adelaide, Australia. The degree was awarded in May, 1996.

Training:

1996-1997: Post-Doctoral Fellow, Department of Pathology, Columbia University College of Physicians and Surgeons, New York, NY.

1997- 1999: Post-Doctoral Fellow, Department of Urology, Memorial Sloan-Kettering Cancer Center.

1999- 2000: Research Associate, Department of Cancer Biology, The Cleveland Clinic Foundation.

October 2000 - present: Project Scientist, Department of Cancer Biology, The Cleveland Clinic Foundation.

Scientific and Medical Societies:

Member of the American Association for Cancer Research (1997-present).

Patent Applications: "DNA Encoding the Prostate-Specific Membrane Antigen-Like Gene and Uses Thereof." #D6230; O'Keefe, D.S. and Heston, W.D.W. (Domestic & International Application).

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30. **O'Keefe, D.S.,** Bacich, D.J. and Heston, W.D.W. Comparative Analysis of the Prostate-Specific Membrane Antigen (PSMA) versus a Prostate-Specific Membrane Antigen-*Like* Gene *(submitted)*.
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Book Chapters

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Comparative Analysis of Prostate-Specific Membrane Antigen (PSMA) versus a Prostate-Specific Membrane Antigen-*Like* Gene

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ABSTRACT

Prostate cancer is the leading cause of cancer and second leading cause of cancer death among American males. Although localized prostate tumors are slow growing and can be treated by radiotherapy or surgery, once the tumor is hormone refractory and has metastasized there are few options for the patient. Currently PSMA is showing promise both as an effective imaging agent and therapeutic target for metastases. First generation antibodies against PSMA are used for the FDA approved Prostascint™ monoclonal antibody scan. In addition, second generation antibodies are being developed for therapeutic targeting as well as imaging (1). However, there have been reports describing RNA expression of PSMA or a very similar gene in non-prostatic tissues including kidney, liver and brain. As we had previously showed that the PSMA gene had been subject to a duplication several million years ago, we set out to determine if this non-prostatic expression was due to expression of the PSMA or another gene. Here we describe the cloning and characterization of the duplicate gene which we have called the PSMA-Like gene. The PSMA-Like gene maps to the Schizophrenia disorder type II locus on 11q14.3, and possesses 98% identity to the PSMA gene at the nucleotide level. The PSMA-Like gene is expressed in kidney and liver and utilizes a different promoter to the PSMA gene.

INTRODUCTION

Prostate-Specific Membrane Antigen (PSMA), also known as Folate Hydrolase 1 (FOLH1) is an ideal potential target for use in determining patient management and therapeutic strategies against prostate cancer. It is highly expressed in both localized and metastatic prostate cancer (2-4). Furthermore, PSMA is a type II membrane protein, with the majority of the protein located outside the cell readily available for therapeutic targeting or clinical imaging or other diagnostic-type assays (5). In addition, it now seems that therapeutic targeting of the PSMA molecule may have additional advantages; PSMA expression has been found in the endothelial cells of tumor vasculature of almost all types of tumors examined to date, including bladder, renal, breast and lung carcinomas (4, 6, 7). No PSMA expression has been reported in normal established vasculature. As such, a therapeutic approach targeted at PSMA could have broad implications for the treatment of many types of solid tumors. Accordingly, several groups are now attempting to utilize PSMA as a clinical and treatment target (1, 8-10). Clinical trials using radiolabeled antibodies to the external domain of PSMA have shown excellent results for imaging primary tumors and distant metastases not previously detected by conventional methods (10). However, although PSMA is very highly expressed in normal and cancerous prostate, there are other tissues in the body that express low levels of PSMA or a similar mRNA including kidney, liver and brain (11, 12). In order to use PSMA as a target, we wanted to know if this non-prostatic expression was in fact from the PSMA gene, and if it was not, what gene was expressed in these tissues. We recently mapped the PSMA gene to human chromosome 11p11.2, and a PSMA-*like* gene to chromosome 11q (13). Both genes are the result of a

genetic duplication that occurred 14 million years ago (13, 14). In order to determine where each of these two genes are expressed, we have cloned the PSMA-*Like* gene and demonstrated methods to distinguish the two genes at the DNA, mRNA and protein levels, which will aid in evaluating diagnostic and therapeutic strategies targeting PSMA.

Materials and Methods

Fine Mapping of the PSMA-Like gene

Primers that specifically amplify the PSMA-Like gene were used to screen the Genebridge 4 Radiation hybrid panel (Research Genetics). The primers were: 5' gccttcattttcagaacatctcatgcat 3' and 5' gtccatataaactttcaagaatgtg 3' ; the primer sequences were based on PCR sequencing/comparison of intronic regions of the PSMA-Like gene by amplification of somatic cell hybrids as described below. Conditions were 35 cycles of 94°C 30", 60°C 30", 72°C 1'. The results were analyzed using the server at http://www-genome.wi.mit.edu/cgi-bin/contig/rhmapper.pl.fordfay.

Genomic Sequence of the PSMA-Like Gene

Sixteen sets of primers were designed based on the genomic sequence of the PSMA gene (accession AF007544) as shown in Table 1. Briefly, 100ng of DNA from a somatic cell hybrid containing the distal portion of human chromosome 11q (NA11936 from Coriell Cell Repositories, Camden, NJ) was amplified for 35 cycles using the following primers. Conditions were: 95°C 30", the annealing temperature indicated in Table 1 for 30" followed by extension for 1' at 72°C. In some cases as indicated, it was necessary to add DMSO to the reaction. Reactions were carried in a total volume of 50µl out using 1 U of Expand High-fidelity Taq DNA polymerase in Buffer 2 supplied by the manufacturer (Roche, Indianapolis, IN), 0.2mM of each dNTP and 150ng of each primer. PCR products were purified using the Wizard PCR Purification kit (Promega, Madison, WI) and directly sequenced on an ABI prism 3100 Genetic Analyzer. DNA from a somatic cell hybrid containing human chromosome 11p (NA11944) was used as a positive control.

RT-PCR

RNA was either made from cell lines using Trizol (Invitrogen, Carlsbad, CA), or obtained from Clontech (BD Biosciences, Palo Alto, CA). The bone marrow endothelial cell line (BMEC) was a kind gift from Dr. Malcolm Moore, Sloan-Kettering Institute for Cancer Research, NY,NY). RT-PCR was carried out using the Superscript Preamplification Kit (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. PCR to differentiate the PSMA and PSMA-Like genes was carried out using 2ul of the cDNA reaction as template. The primers used were: 5' acagatatgtcattctgggaggtc 3' and 5' actgtgatacagtggatagccgct 3'. Initial denaturation was for 4 mins at 95°C followed by 35 cycles of 95°C 30", 60°C 30", 72°C 1'. Ten µl of PCR product was digested with 10 U of *EcoRI* (New England Biolabs, Beverly, MA) in a total volume of 15µl at 37°C for 2 hours. Products were resolved on a 1.5% agarose gel.

Cloning of the PSMA-Like cDNA

A liver cDNA plasmid (bacterial) library (Invitrogen, Carlsbad, CA) was screened as previously described (15) using a probe generated via PCR using the following primers : 5' gtttataaaatcctccaatgaagc 3' and 5' gagcttctgtgcatcatagta 3' (exons 2-7 of PSMA) or we used a probe spanning exons 10-16, generated by the same primers as used for the RT-PCR described above. Three prime RACE was carried out using the 3' RACE system (Life Technologies, Gaithersburg, MD), with the primer 5' ttgaggtgttcttccaacgac 3' and a PSMA-Like specific primer 5' gacaaaagcaacccaatattg 3'. The cDNA sequence has been deposited in Genbank under accession number AF261715.

Northern Analyses

Multiple Tissue Northern blots were obtained from Clontech. Hybridization with the hPSM-350 riboprobe (16) was carried out overnight at 56°C, followed by washing for one hour in 0.1 x SSC, 0.1% SDS at 65°C as previously described (15). Exposure was carried out for 5 hours at –80°C. The PSMA-Like (Not1/Sal1 digest of the original clone in pSPORT) and β-actin probes were prepared using random-hexamer labeling (Invitrogen, Carlsbad, CA). Hybridization with the PSMA-Like probe or the β-actin probe (Clontech) was carried out overnight at 42°C in 50% formamide (Hybrisol I, Intergen, Purchase NY), followed by washing at 42°C for 15 minutes in 0.2 x SSC, 0.1% SDS. Quantitation of the relative amounts of PSMA expression in various tissues was carried out using the Image-J program with the gel analyzer plugin available from the NIH website http://rsb.info.nih.gov/ij/.

Regulation of the PSMA-Like Gene

The region of the PSMA-Like gene that corresponds to the PSMA enhancer was cloned and sequenced using PCR with the following primers that incorporate artificial *BamHI* restriction sites (underlined) to amplify NA11936 DNA: 5' cgcggatccgccttctaaaatgagttggg 3' and 5' cgcggatccggctactacataagtataagtc 3' which produces a product of 1648 bp. The PCR product was cloned into the *BamHI* site of the pGL3-B-PSM luciferase reporter vector containing the PSMA promoter and activity of the enhancer determined as we have previously described (17), with the addition that MDA PCa2b cells (ATCC, Rockville, MD) were maintained BRFF-HPCI Catalog # SF-30 (Biological Research Faculty Facility, Ijamsville, MD) supplemented with 15% fetal calf serum (Invitrogen). The sequence of the PSMA-Like enhancer region has been deposited in Genbank, accession number AF480875.

Transient Transfections and Enzyme Activity Analysis

The PSMA-Like gene was excised from pCMV·SPORT using *EcoRI and BamHI* and subcloned into the same sites in the pIRES-neo (Clontech) vector. The PSMA/PSMA-Like hybrid was made by excising 1211 nt from PSMA-neo with *XcmI* and *EcoRV* and subcloning the fragment into the same sites in PSMA-Like-neo. PC3 prostate cancer cells were plated in six-well dishes so that they would be 90% confluent the following day. The cells were then transfected with 4.5 ug of either PSMA-Like-neo, PSMA-neo or PSMA/PSMA-Like hybrid-neo and 900ng of pAdVantage (Promega) using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. The samples were harvested 30 hours post transfection using 120ul of lysis buffer per well (50mM Tris-HCl ph 7.5, 0.5% triton X-100) and the protein quantitated using BCA Protein Assay Reagent (Pierce, Rockford, IL). Ten ug of protein was incubated at 37°C for 1 or 20 hours in a total volume of 100ul of lysis buffer containing 4μM N-acetyl-L-aspartyl-L- (glutamate-3,4-3^{H}) (Perkin Elmer Life Sciences, Boston, MA). The reaction was stopped by the addition of an equal volume of 0.5M NA_2HPO_4. Liberated glutamate was separated using ion-exchange chromatography as previously described (18). Briefly, half the reaction was layered over a Poly-Prep prefilled chromatography column packed with AG-1-X8 formate resin, 200-400 mesh (Biorad, Hercules, CA) that had been washed with two volumes of distilled water. The column was washed with 2mls of 1N formic acid, and the free-glutamate eluted in 2.5mls of 1N formic acid followed by measurement using scintillation spectrometry.

Immunoblotting

Western blotting for PSMA was carried out by transferring 25ug of protein to PVDF membrane as described elsewhere (15). The blot was probed with the Cyt-351 antibody (a

gift from Cytogen corporation, Princeton, NJ) or PM2M-440 (a gift from Hybritech Incorporated, a wholly owned subsidiary of Beckman Coulter, Inc., San Diego, CA) and exposed using the ECL system (Amersham Biosciences, Piscataway, NJ). Other antibodies used in studies described here are J591 (a kind gift from Dr. Neil Bander, Weill Medical College of Cornell University, NY, NY) or PEQ-226 and PM1T-485 (kindly supplied by Hybritech Incorporated, San Diego, CA).

Results

Fine Mapping of the PSMA-Like gene

PSMA-Like specific primers were designed based on PCR amplification and sequencing of a region of the PSMA-Like gene that is homologous to part of the first intron of the PSMA gene. These primers were then used to screen the Genebridge 4 radiation hybrid panel, placing the PSMA-Like gene 8.99 cR from CHLC.GATA45H10 and 0.40 cR from WI-6090 (LOD>3).

Genomic and cDNA Sequence of the PSMA-Like Gene

We were unable to screen a cDNA library to obtain the PSMA-Like cDNA clone, as we were unsure which tissue(s) it was expressed in, or indeed if it was expressed. Subsequently, we designed 16 sets of primers to the gene, the sequence of each set based on the intronic regions flanking the exons of the homologous PSMA gene. Using DNA from somatic cell hybrids retaining human chromosome 11q, we were able to generate products comprising each exon of the gene using the primers described in Table 1, except for exon 1. All exons sequenced conformed to the GT-AG intron-exon boundary rule. The differences that we were able to determine at the genomic level are summarised in Table 2.

In exon 12, a g→t change alters an *EcoRI* restriction enzyme site that is present in the PSMA gene so that it is no longer cleavable in the PSMA-Like gene. To confirm this finding, we amplified exon 12 in 18 unrelated people and somatic cell hybrid DNA containing either 11p or 11q. All individuals exhibited three bands after digestion, indicating the presence of one non-cleavable and one cleavable gene, confirming the g→t

difference was not a polymorphism in the 11q gene (data not shown). To determine which tissues the PSMA-Like gene might be expressed in, we carried out RT-PCR using primers spanning exons 10-16, followed by restriction enzyme digestion with *EcoRI* (Fig. 1).

Using this *EcoRI* non-cleavable site as a "sequence tag" for the PSMA-Like gene, we were able to determine that liver and kidney showed a restriction enzyme banding pattern that corresponded to expression of *both* the PSMA and the PSMA-Like genes, while the other positive tissues tested showed expression only of the PSMA gene. Next, we screened a 2.3 x 10^6 colony forming units from a liver cDNA library using a probe to exons 2-7 of the PSMA cDNA sequence. Subsequent clones were digested with *EcoRI* in order to exclude PSMA clones from further analysis. Only two PSMA-Like clones could be identified this way although we found 26 full-length PSMA clones and two partial PSMA clones (beginning in exon 2 and 3 respectively). Both PSMA-Like clones that we isolated began in a region corresponding to intron 5 and exon six of the PSMA gene. We next screened the library again, this time using a probe generated from the PCR product spanning exons 10-16 of the PSMA gene. This generated a further 12 PSMA-Like clones (and no more PSMA clones), the most 5' sequences of which corresponded to the same intron 5 of the PSMA gene. We confirmed the 3' end of the gene using 3' RACE and specific primers based on the PSMA-Like gene sequence. The three longest clones from the library were sequenced, and the complete nucleotide and deduced amino acid sequence compared to that of the PSMA gene (Fig. 2). The sequence has been deposited in Genbank, accession AF261715. The longest open reading frame of the PSMA-Like gene is homologous to the reading frame of PSMA. In addition, *in vitro* translation of the longest clone yielded the

expected 46kD protein (data not shown). At the mRNA level PSMA-Like is 98% homologous to PSMA, and the protein shows 97% identity and 98% similarity to PSMA in the translated region. It should be noted however, that the expected size of PSMA-Like *in vivo* is 46kD, while PSMA is 100-120kD after glycosylation of its 84kD core.

Expression Pattern of the PSMA and PSMA-Like Genes

To determine what tissues express PSMA while avoiding detection of the PSMA-Like gene, we used a probe from the first three exons of PSMA which are not found in the PSMA-Like cDNA sequence (probe p350). Northern analysis confirmed expression of PSMA in the prostate, brain, kidney, small intestine, liver and spleen (Figs. 3A and 3B). After prostate, the next five highest expressing regions were all from the brain, and the other tissues were all at levels less than 10% of that of PSMA in normal prostate (Table 3). Similarly, western analysis using the Cyt-351 and PM2M-440 antibodies showed protein expression in the hippocampus and amygdala compared to that seen in the prostate cancer cell line MDA PCa2b (Figs. 4a and b). The Cyt-351 antibody binds to the intracellular region of PSMA that is deleted from the PSMA-Like gene (19), while PM2M-440 binds to a region within amino-acids 135 and 173 of PSMA (pers. communication with Harry Rittenhouse, Hybritech Incorporated), which is not found in the PSMA-Like cDNA.

PSMA mRNA expression was either neglible or not detected in thymus, testis, ovary, colon, leukocytes, heart, placenta, lung, muscle and pancreas. In addition, we cloned and sequenced PSMA expressed in tumor neo-vasculature and confirmed that it was not PSMA-Like. However the clone sequenced did contain two changes at nt 1784 (G→A;

Gly→Asp) and nt 1817 (A→G; Asn→ Ser). To determine the relative expression of the PSMA-Like gene, we next probed with its cDNA in entirety (Fig. 5a-d). This would detect both PSMA (2.7kb) and PSMA-Like (2.0kb) mRNA. PSMA-Like was expressed at similar levels to the PSMA gene in adult kidney and liver, but not in any other tissues including fetal kidney, liver, brain and lung. Interestingly, PSMA is expressed in fetal liver and kidney, and also in adult trachea and spinal cord.

Regulation of the PSMA-Like Gene

We cloned a prostate-specific enhancer from the third intron of the PSMA gene (17, 20). As we had shown that the PSMA-Like gene was not expressed in the prostate (Fig. 1 and data not shown), we were interested to compare the sequence of the two genes "enhancer" regions. PCR using primers homologous to the PSMA enhancer were used to amplify DNA for sequencing from the 11q-containing hybrid. Suprisingly, the sequence of the two intronic regions is 99.3% identical. Because the minor differences between the two regions might alter novel prostate-specific transcription factor binding sites, we tested the PSMA-Like enhancer region for its ability to drive luciferase reporter gene expression in combination with the PSMA promoter (Fig. 6).

The PSMA-Like enhancer was able to drive luciferase expression equally as well as the PSMA enhancer in C4-2 prostate cancer cells. In addition, like the PSMA enhancer, there was no activity in the breast cancer derived cell line MCF-7. The PSMA-Like enhancer showed approximately equal activity in both the C4-2 and MDA PCa2b prostate cancer cell

lines, however the PSMA enhancer repeatedly showed a 2.5 fold increase in activity in the MDA PCa2b cell line over its expression in the C4-2 cell line.

As the enhancer of the PSMA-Like gene was able to activate prostate-specific gene expression, we wanted to examine the region of the PSMA-Like gene that corresponded to the PSMA promoter, but had been unable to amplify any region of it or exon 1. A BLAST comparison with the high throughput genomic sequence database revealed homology of the PSMA-Like gene with a contig of 27 unordered pieces (Genbank accession AC024234). BLAST analysis of this contig against the promoter region and exon one and two of the PSMA gene in an attempt to identify a PSMA-Like promoter showed a deletion in AC024234 in the region corresponding to approximately 500nt upstream of the PSMA transcription start site, the entire first exon, and the first 371nt of intron one. To confirm this result, we designed primers to either side of the deletion, and used them to amplify DNA from five unrelated people. As the primers could amplify both PSMA and PSMA-Like, we expected to see approximately a 330bp product generated in all individuals if the deletion had occurred, and a 1.5kb band from the PSMA gene. All five DNAs produced a 330bp band, while the 11p hybrid produced a 1.5kb band (from the PSMA gene) and the 11q hybrid a band of approximately 330bp, verifying that deletion of the region corresponding to the promoter and exon one of PSMA had been deleted in the PSMA-Like gene (Fig. 7). No 1.5kb band from the PSMA gene is visible in the human DNA lanes, probably due to preferential amplification of the smaller product.

To see if there is a promoter close to the transcription start site of the PSMA-Like gene, we analyzed 550 nt of sequence upstream using the promoter prediction program at http://www.fruitfly.org/seq_tools/promoter.html, which uses a neural networking method to predict promoter regions. A promoter was predicted 150 nt upstream of the 5' end of the longest clone obtained. In addition, there is a CCAAT box 161 nt and a TATA box 28 nt from the transcription start site.

Enzymatic Activity of the PSMA-Like Gene

PC3 cells were transfected with PSMA or the PSMA-Like gene and assayed for NAALADase activity. Despite the few amino acid differences between the two genes and the fact that the PSMA-Like gene possess the enzymatic domain of PSMA (21), the PSMA-Like gene appeared to have no activity. However, we were unable to confirm PSMA-Like protein expression. This might have been because either the anti-PSMA antibodies we were using (J591, PM1T-485 and PEQ-226) did not bind to PSMA-Like or due to low expression/protein stability. Repeatedly in transient transfections the majority of the PSMA-Like transfected cells were dead after 24 hours, although all other transfections only suffered up to 20% cell death. So that we could be sure that we could detect PSMA-Like expression, and because the lack of enzyme activity might be due to loss of the protease-associated domain that is found in a number of diverse proteases and as such might be structurally or otherwise important (a.a. 160-280 of PSMA), we made a hybrid PSMA/PSMA-Like construct. The first 307 amino-acids of the hybrid protein are the first 307 a.a. of the PSMA protein, while the rest of the protein is PSMA-Like in its

entirety. As the Cyt-351 antibody binds the internal domain of PSMA (19), it detects expression of the PSMA/PSMA-Like hybrid protein. Suprisingly, this construct did not have enzymatic activity in PC3 cells, although the transfection with PSMA did (Figs. 8 a, b and c).

Discussion

In order to determine the origin of the so called "non-prostatic" expression of PSMA, we cloned and characterized the expression pattern of the PSMA-Like gene. The PSMA and PSMA-Like genes arose from a duplication event of the original gene approximately 14 million years ago (14, 22). The site of the original gene was most likely 11q14.3, as this region has conserved synteny with the location of the single murine PSMA homolog, folh1 at 7D1-2 (23). The finding that the PSMA-Like gene maps to the schizophrenia disorder type II locus is particularly interesting as a disruption in the NAALADase activity of PSMA has been implicated in the pathogenesis of schizophrenia (24).

Presumably the loss of the genomic DNA segment in the PSMA-Like gene that corresponds to the promoter and exon one of PSMA occurred subsequent to the duplication event. The similarity between the PSMA and PSMA-Like genes is remarkable, yet the very few differences between them allows us to learn more about the biology of PSMA. Despite the fact that the "enhancer" regions of the two genes are 99.3% identical, the PSMA enhancer is more than twice as active than the PSMA-Like enhancer combined with the PSMA promoter in MDA PCa2b cells. However, both enhancers work equally well in C4-2 cells. This suggests that some of the few sequence differences correspond to important enhancer factor binding sites for factors that are present only in MDA PCa2b cells. It has previously been reported that a 330 bp core region contributes most of the activity of the PSMA enhancer (20). Comparison of the two enhancer sequences reveals that two of the differences abolish binding sites for ATF/CREB and CEBP although there could be other sites as yet unrecognized that are also altered. Dissection of the factors

controlling PSMA expression is important as we and others have been utilizing the PSMA enhancer to develop gene therapy strategies (17, 25).

As the PSMA-Like enhancer is able to drive expression of the PSMA promoter, and the PSMA enhancer is able to drive expression of minimal heterologous promoters such as TK (20), we might expect to see prostatic activation of the native PSMA-Like promoter. However, this clearly does not occur as we do not see expression of PSMA-Like in the prostate. One possible explanation for this is that an insulator region exists between the enhancer and the PSMA-Like promoter, blocking it from becoming active in prostate-derived tissue (26). In addition, although the region corresponding to the PSMA-Like promoter in the PSMA gene is almost identical, it is not activated by the PSMA enhancer or we would expect to see a 2.0kb mRNA band in prostate tissue via northern analysis.

Loss of the region corresponding to exon one of the PSMA gene from the PSMA-Like gene might also explain the lack of enzymatic activity of PSMA-Like. Exon one of PSMA encodes for the single transmembrane domain. As the PSMA-Like cDNA sequence lacks a transmembrane domain, we expect it to be located within the cytosol, and therefore not subject to the glycosylation that is undergone by the PSMA protein (27). Barinka et al. recently reported that glycosylation of PSMA is indispensable for enzymatic activity (28). However, although analysis of expression of the chimeric PSMA/PSMA-Like protein via western blotting appeared to produce a correctly glycosidated protein, it was still enyzmatically inactive. The PSMA/PSMA-Like chimera only has three amino acid differences to PSMA that are not conserved in other species homologs of PSMA that are

enzymatically active. One of these differences is found at the carboxyterminal of the protein, presumably away from the catalytic region a.a. 728 (E→D) (21). However the other two of these differences (a.a. 398 I→T and a.a. 437 E→D based on the PSMA a.a. sequence) correspond to the catalytic region of PSMA, close to the amino acids responsible for zinc ligand binding and catalysis (a.a. 424 and 425). Site-directed mutagenesis of residues in the vicinity of the zinc ligands has been shown to dramatically reduce the activity of PSMA (29). Therefore it is likely that one or both of these amino acid changes found in the PSMA-Like gene alters the local structure of the enzyme, rendering it inactive and as such these residues might be good candidates for targeting by small-molecule inhibitors. Moreover, Devlin et al. reported that a polymorphism exists in the PSMA gene which alters a single amino-acid (H475Y) resulting in a 50% reduction in the folate hydrolase activity of PSMA (30). While this polymorphism is present in PSMA at an allele frequency of 0.04, it is present in 100% of PSMA-like alleles we have examined, and perhaps in combination with the other amino acid changes results in a protein with no enzymatic activity even when glycosidated correctly.

The aim of this study was to analyze the possible expression of PSMA in non-prostatic tissues. We have shown here that PSMA is in fact expressed in a number of non-prostatic tissues. This expression is not due to other hypothetical or known homologs of PSMA as described in the EMBL database, as our p350 probe would either not detect these mRNAs, or would bind to an mRNA of a significantly different size to PSMA (31, 32) and observations from EMBL database (http://www.ensembl.org/Homo_sapiens). In addition, it should be noted that although there are two "homologs" of PSMA shown on

chromosome 2 in the EMBL database, we have tested the possible existence of these using PCR of somatic cell hybrids containing human chromosome 2, and concluded that they are the result of artefact and in fact do not exist (data not shown). Also, we and others have mapped PSMA using BAC arrays and somatic cell hybrids to 11p11.2, but the EMBL database maps PSMA to the 11q side of the centromere (13, 14). As such caution should be excercised when using the genome database as a resource.

The reasonably high expression of PSMA mRNA in the various regions of the brain, the spinal cord, liver and kidney is of significant concern and needs to be addressed when designing therapeutic strategies utilizing PSMA as a target. However the brain and spinal cord are protected by the blood-brain barrier. In addition, there is no immunohistological evidence of PSMA protein expression in the brain or liver, despite several studies of these tissues (reviewed in Tasch et al. (33)). The same studies have shown positive staining for PSMA in the kidney, where it is expressed weakly in a subset of proximal tubule cells. It is possible that PSMA protein expression is regulated post-transcriptionally, so that the amount of mRNA present is not an indicator of actual protein. Prostate cancers contain over 1000 fold greater levels of PSMA than found in liver or brain as determined quantitatively by RIA (12). Most importantly extensive imaging studies (34) and phase one trials using cytotoxic radiolabeled, humanized antibodies against PSMA (35) have shown specificity for prostate, prostate cancer, and the neovasculature of other solid tumors including renal cell carcinoma. With both treatments there was a low frequency of side-effects, and in addition it seems the treatment with the antibody conjugated to β-emitters might be in the therapeutic range, as some of the patients with prostate cancer had as much

as an 85% reduction in PSA. Consistent with a blood-brain barrier effect, immunotargeted antibody approaches with radiolabeled antibodies against PSMA used for imaging do not show localization in the brain (36). It is also heartening that there have been no reports of tissue injury in phase II trials stimulating the immune system against PSMA. Indeed, the trials have shown little if any toxicity, but a positive response rate in 30% of the patients (37).

Finally, we have cloned and characterized a gene that is highly homologous to PSMA, and determined ways to distinguish the two genes at the DNA, mRNA and protein levels. PSMA can be a useful clinical target for prostate cancer, however both the presence of the PSMA-Like gene and expression of PSMA in other tissues should be taken into consideration when designing diagnostic and therapeutic strategies. In addition, the small but significant differences between the evolutionary twins, PSMA and PSMA-Like, allows us to learn more about the function and regulation of PSMA.

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Exon	Bases	Sense Primer	Sense Primer Sequence	Anti-sense Primer	Antisense Primer sequence	PCR product size expected
1	2488-2863	2529	tctctctctcgctcggattgg	2863	cgaagaggaagccgaggag	335 n/a
2	4994-5099	4341	tgtttctggccgcctatgcg	5254	agtatagtcctcctcagatg	914*
3	10726-10912	10630	caaagtacttttgtgtaactctgc	11082	cataggaaagtagttgacacgg	452#
4	18275-18376	18157	cctgaaggattcattcaccctc	18457	gacccttaattatcggctgaaca	300##
5-6	24400-25500	24323	atgtccaacagtccccatgcag	25593	gacatgcttagtccattgtacc	1270##
7	27927-28020	27871	gaaccgtttgaatgaaactgag	28058	ttacccaaatagccatccatgg	187*
8-9	35216-36281	35127	gcagatgctcaataagtgaatcc	36334	ccagcacataacagttacttgatc	1207#
10	37697-37816	37619	tagatgctattgagtcgtttgc	37867	aaactgagactcagataggctg	248#
11	39896-39978	39825	ctgggcttggtagtgtcctggg	40045	gcttggcaaacaagtcctggctac	220**
12	41911-41974	41792	tgtcgttaatatgggtcagctc	42035	ttaactagactgctgctcctag	243*
13	46402-46469	46317	tggtaggaatttagcagtggtc	46687	gatgctactaatgggctacctc	370**
14	53129-53220	53053	cttctggttaatggacatctag	53264	caatcccacactgaattcagtg	211♦
15	54364-54454	54278	agaatggggtttagtttaatgg	54536	tgagtcacttttggagtcag	258*
16-17	56661-57307	56614	ttgtaagctatccctataagag	57393	agttcagcaacagtcatgttag	779♦
18	62423-62515	62305	gggtggtcctgaaaccaatccc	62553	gtgatattacagaaaggagtc	248**
19	64209-64518	64127	atccaggaattgcagagtgctc	64586	ttcagttttaatccatagggag	459**

Table 1: Primer sequences and PCR conditions used to amplify the PSMA-Like gene. All sets of primers generated the indicated size product, except for exon 1 which was not able to be amplifed. Annealing temperatures are indicated; *55°C, **57°C, #58°C, ##60°C. ♦ indicates an annealing temperature of 55°C in the presence of 5% DMSO.

Exon # in PSMA	Nucleotide changes PSMA⇒PSMA-like	Amino-acid change PSMA⇒PSMA-like
1	Not present	n/a
2	No change	No change
3	nt. 630 t→a nt. 584 t→c nt. 594 a→t	Threonine→Threonine Valine→Alanine Alanine→Alanine
4	nt. 739 c→t	Proline→Serine
5	nt. 777 c→t nt. 787 t→c nt. 877 g→a	Glycine→Glycine Tyrosine→Histidine Glycine→Arginine
6	nt. 948 c→a nt. 993 t→c nt. 1023 g→t	Serine→Serine Aspartic acid→Aspartic acid Glutamine→Histidine
7	nt. 1092 t→c nt. 1103 g→a nt. 1150 a→g	Tyrosine→Tyrosine Arginine→Glutamine Isoleucine→Valine
8	nt. 1237 c→t	Proline→Serine
9	nt. 1320 a→g	Threonine→Threonine
10	nt. 1454 t→c	Isoleucine→Threonine
11	No Changes	No Changes
12	nt. 1572 g→t	Glutamic acid→Aspartic acid
13	nt. 1665 g→a nt. 1684 c→t	Proline→Proline Histidine→Tyrosine
14	No Changes	No Changes
15	No Changes	No Changes
16	nt. 2099 g→a nt. 2140 g→t	Serine→Asparagine Valine→Leucine
17	nt. 2172 g→a nt. 2202 t→c	Lysine→Lysine Serine→Serine
18	nt. 2239 g→t and nt. 2241 a→g nt. 2314 g→a	Valine→Leucine Arginine→Arginine
19	nt. 2442 a→t nt. 2459 a→c nt. 2531 a→c nt. 2534 c→t nt. 2562 AG is deleted in PSMA-like nt. 2571 c→a nt. 2572 g→a	Glutamic Acid→Aspartic Acid Tyrosine→Serine 3' UTR 3' UTR 3' UTR 3' UTR 3' UTR 3' UTR

Table 2: Nucleotide and inferred amino acid sequence changes relative to the PSMA gene deduced from genomic sequencing of the PSMA-Like gene.

Tissue	Expression (relative to normal prostate)
prostate	100
corpus callosum	59
substantia nigra	35
caudate nucleus	20
hippocampus	17
subthalamic nucleus	12
whole brain	9
kidney	9
thalamus	7
small intestine	6
liver	5
amygdala	4
spleen	2

Table 3: Relative amounts of PSMA in various tissues. The amount of PSMA in various tissues was determined using densitometry of the northern analyses shown in Fig. 5. Expression of PSMA in prostate was arbitarily set at 100. The next five highest expressing tissues are all from the brain, while the next highest expressing organ, kidney, has less than 10% of the amount of PSMA that is seen in the normal prostate. All values were adjusted for β-actin expression (data not shown).

Figure Legends

Figure 1: Expression of the PSMA and PSMA-Like Genes. RT-PCR followed by gene-specific restriction enzyme digestion was used to differentiate expression of the PSMA and PSMA-Like genes in various tissues. The PSMA gene yields two bands of 346 and 208 bps following digestion with *EcoRI*, while the PSMA-Like gene remains uncut at 554 bps. M=100 bp ladder (Gibco), LNCaP=prostate cancer cell line, BMEC=bone marrow endothelial cells, UD=undigested.

Figure 2: Nucleotide and deduced amino acid sequence alignment of the PSMA-Like gene compared to the PSMA gene. Differences in nucleotide sequence are indicated in bold lower-case lettering, while amino acids found in PSMA and not PSMA-Like are indicated in bold capitals above the sequence. The Genbank accession numbers for PSMA-Like and PSMA are AF261715 and M99487 respectively.

Figure 3: Expression of PSMA as determined by northern analysis using a probe that will not detect the PSMA-Like gene. a) Expression of the 2.6 kb PSMA transcript is clearly strongest in the normal prostate, although expression of PSMA can also been seen in other tissues. b) Expression of PSMA is also found in the brain, although the levels vary depending on the region examined. RNA marker sizes are indicated in kb.

Figure 4: PSMA protein expression via immunoblot analysis. a) Using antibody Cyt-351 and b) antibody PM2M-440. Both antibodies are specific for PSMA because they bind to regions of the protein that is missing in the PSMA-Like protein. Densitometry of this blot reveals approximately 40-fold less PSMA protein in the brain tissues amygdala and hippocampus than is seen in the prostate-cancer cell line MDA-PCa2b. The size of the proteins is around 130kD, although it seems that the brain might glycosidate PSMA differently to the prostate.

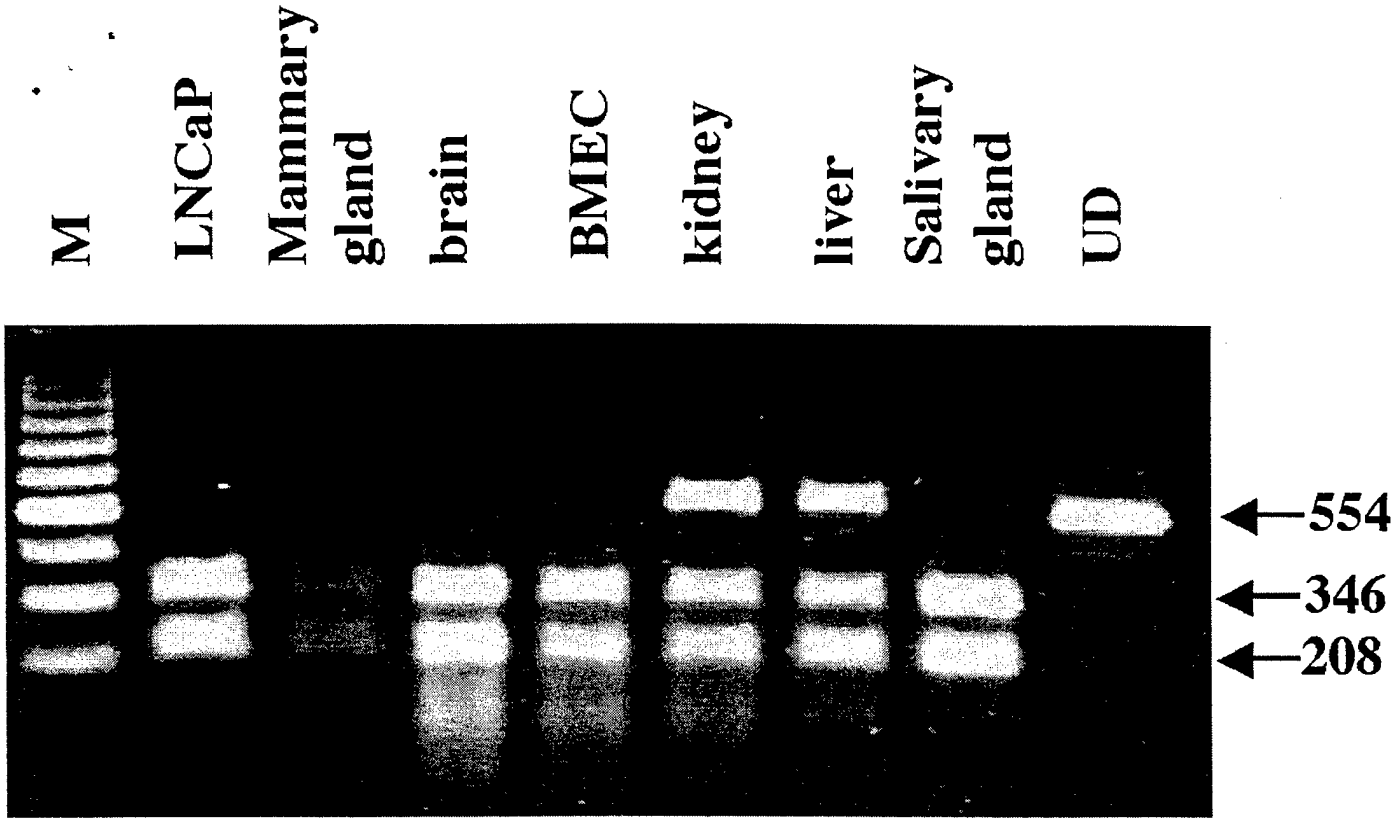
Figure 5: PSMA and PSMA-Like mRNA expression in various tissues as seen via northern analysis. Expression of PSMA vs PSMA-Like was determined by utilizing a probe that detects a 2.7kb band corresponding to PSMA, and a 2.0kb band corresponding to PSMA-Like. a) and b) PSMA and PSMA-Like expression in normal human tissues, c) PSMA expression in brain tissues and d) PSMA expression in fetal kidney and liver. β-actin was used as a loading control.

Figure 6: Comparison of the PSMA and PSMA-Like "enhancer" regions. Potential of the PSMA-Like enhancer to drive a luciferase reporter gene in combination with the PSMA promoter is reported in relative light units after adjusting for transfection efficiency. The PSMA-Like sequence can operate as a tissue-specific enhancer, as evidenced by reporter gene expression in the prostate cancer cell lines MDA PCa2b and C4-2, but not the breast cancer line MCF-7. Interestingly the enhancer has the same activity as the PSMA enhancer in C4-2 cells, while in MDA PCa2b the PSMA enhancer induces more than twice as much reporter gene expression than the PSMA-Like enhancer clones. The black bar indicates the PSMA promoter/enhancer construct, the wavy bars

are two individual clones of the PSMA promoter/PSMA-Like enhancer construct, while the white bar is the PSMA promoter alone. The standard deviation of triplicate experiments is shown.

Figure 7: Deletion of the original PSMA-Like promoter, exon one and part of intron one. PCR analysis was performed using primers based outside the region predicted to be deleted in the PSMA-Like gene. Amplification of the predicted 300 bp band if a deletion had in fact occurred is seen in all five human DNAs (hum 1-5) and DNA from the 11q human-hamster hybrid (11q). Amplification of the 1500 bp band in the homologous region of the PSMA gene is generated from the 11p hybrid (11p). "Ham" indicates the parental hamster DNA of the hybrids.

Figure 8: Enzymatic analysis of PSMA-Like. a) Depiction of PSMA and PSMA prime versus PSMA-Like and the hybrid protein. The hybrid protein consists of the first 307 a.a. of PSMA, followed by PSMA-Like in its entirety. int=internal domain, TM=transmembrane region. b) Immunoblot analysis of PSMA or PSMA/PSMA-Like hybrid expression in transiently transfected PC-3 cells c) NAALADase activity of the transfected cell lines expressed in disintegrations per minute (DPM). Vector alone and hybrid transfected cells have no detectable NAALADase activity. All values have been adjusted for background by subtracting the DPM value of a reaction with no protein added.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208

Figure 1

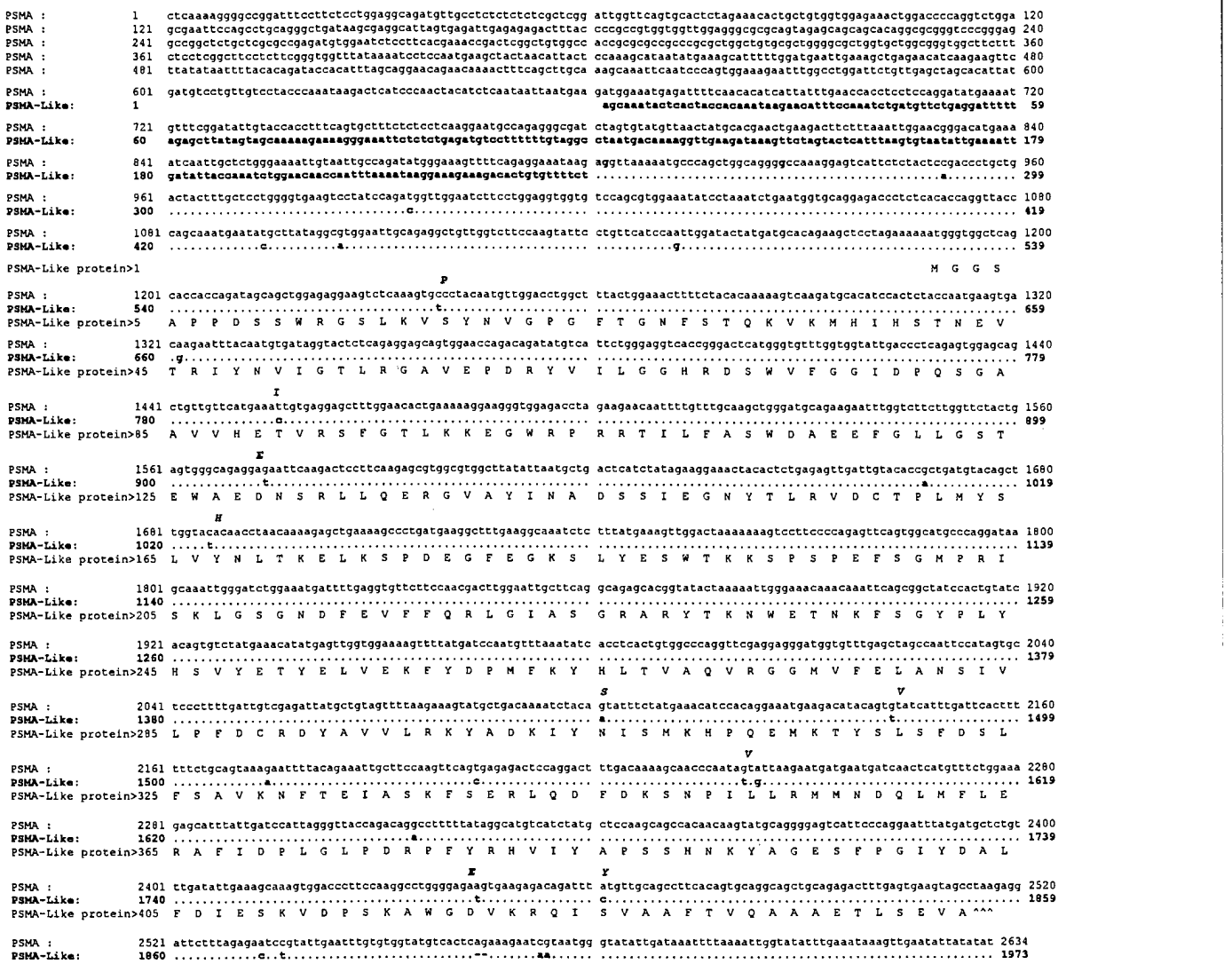
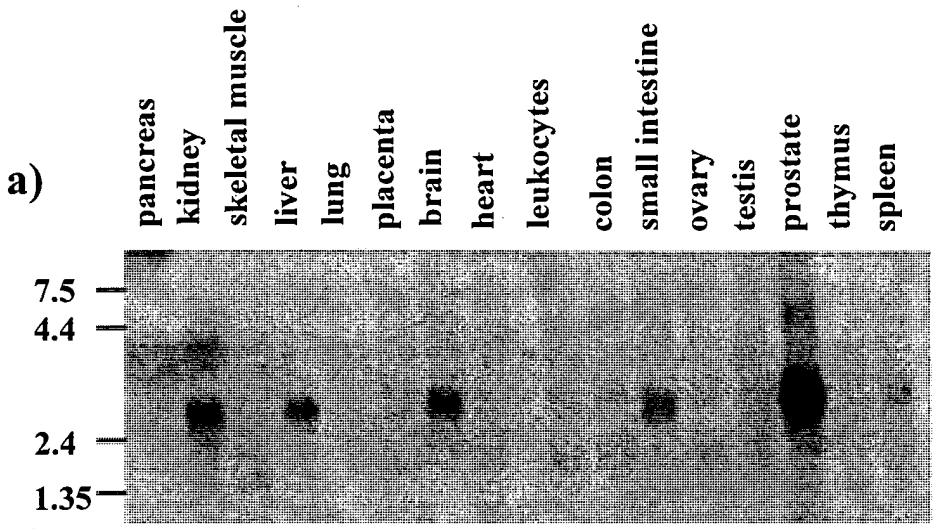


Figure 2


a)
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skeletal muscle
liver
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placenta
brain
heart
leukocytes
colon
small intestine
ovary
testis
prostate
thymus
spleen
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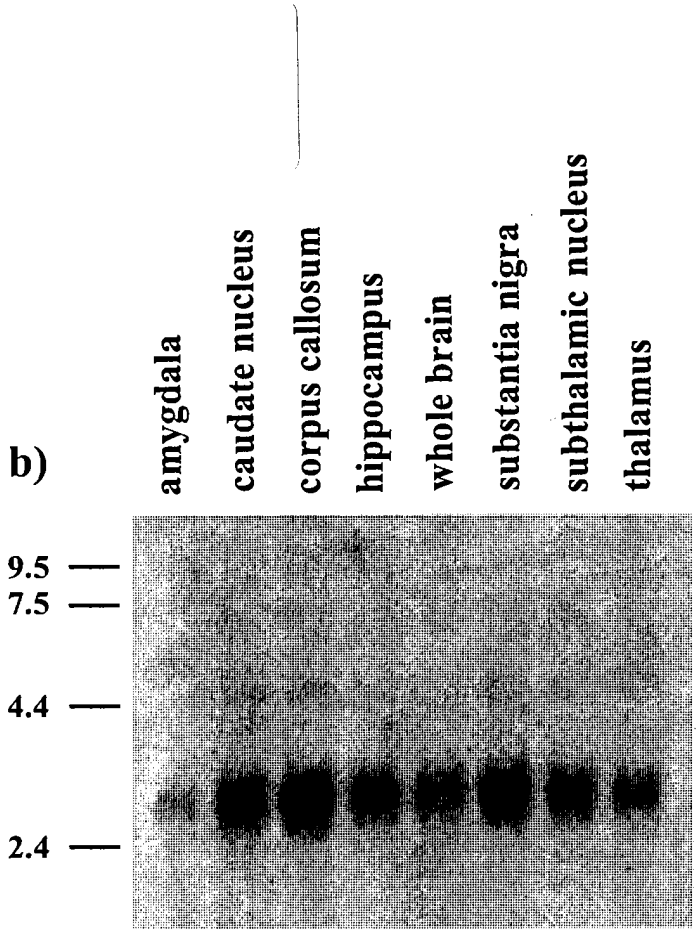
4.4
2.4
1.35

Figure 3 A

Figure 3 B


b)
amygdala
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whole brain
substantia nigra
subthalamic nucleus
thalamus
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4.4
2.4

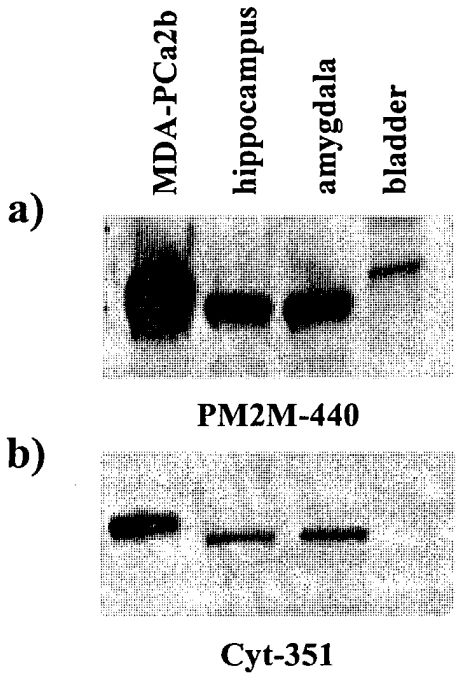
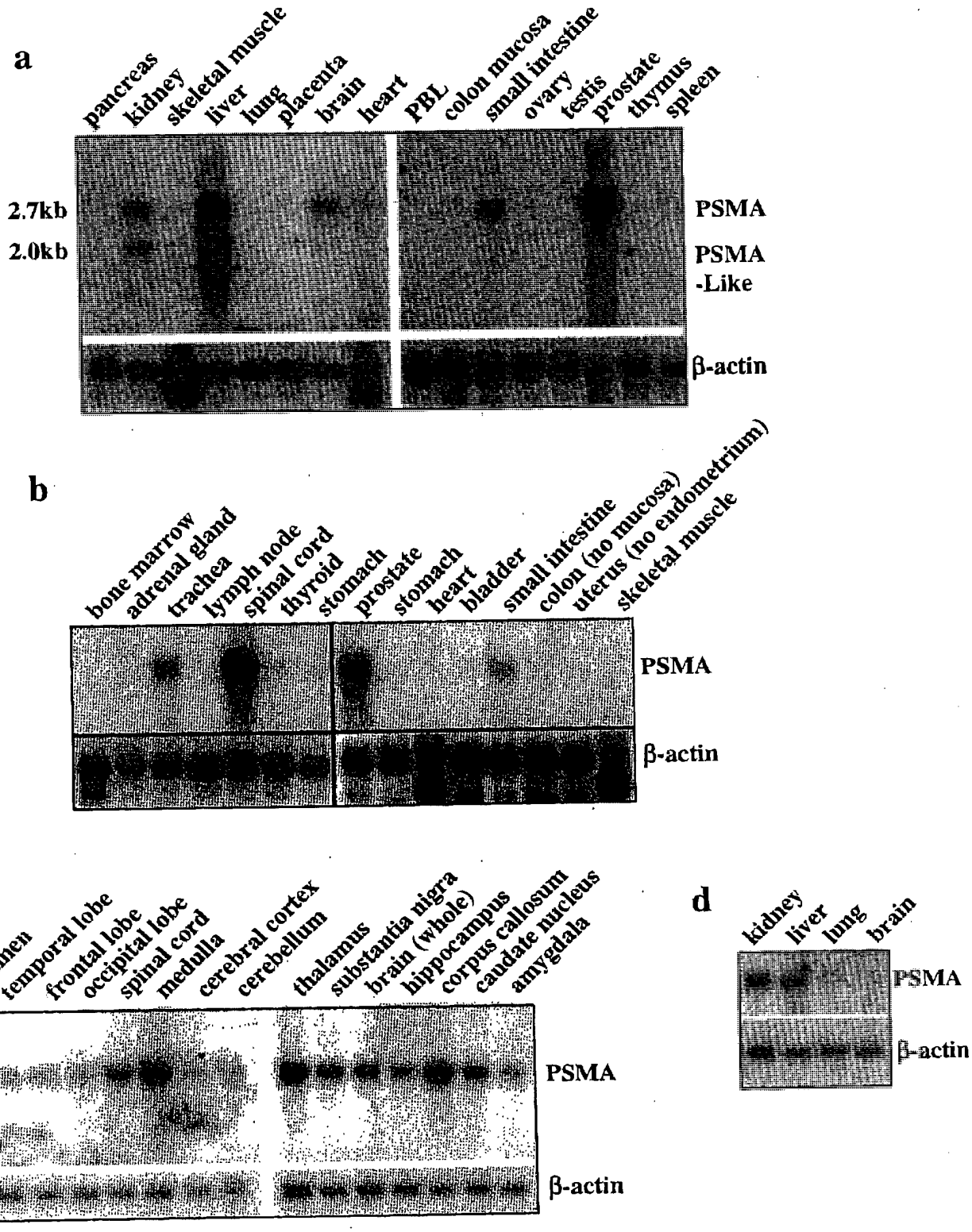


Figure 4

Figure 5


a
pancreas
kidney
skeletal muscle
liver
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placenta
brain
heart
PBL
colon mucosa
small intestine
ovary
testis
prostate
thymus
spleen
2.7kb
2.0kb
PSMA
PSMA -Like
β-actin
b
bone marrow
adrenal gland
trachea
lymph node
spinal cord
thyroid
stomach
prostate
stomach
heart
bladder
small intestine
colon (no mucosa)
uterus (no endometrium)
skeletal muscle
PSMA
β-actin
c
putamen
temporal lobe
frontal lobe
occipital lobe
spinal cord
medulla
cerebral cortex
cerebellum
thalamus
substantia nigra
brain (whole)
hippocampus
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caudate nucleus
amygdala
PSMA
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d
kidney
liver
lung
brain
PSMA
β-actin

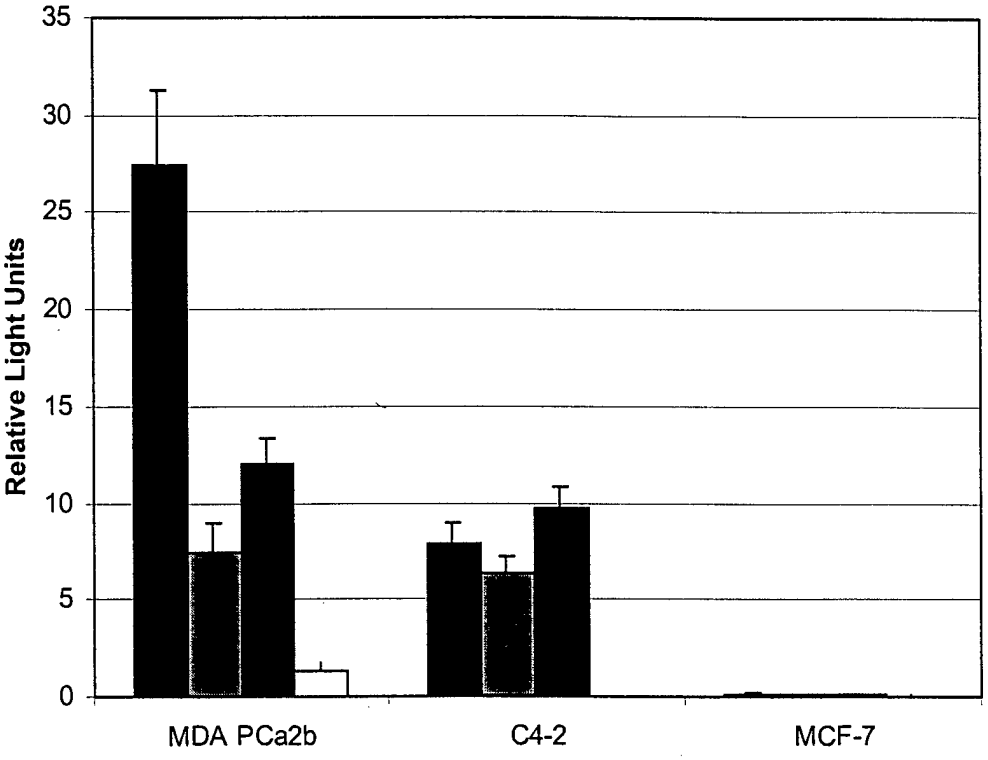


Figure 6

Figure 7

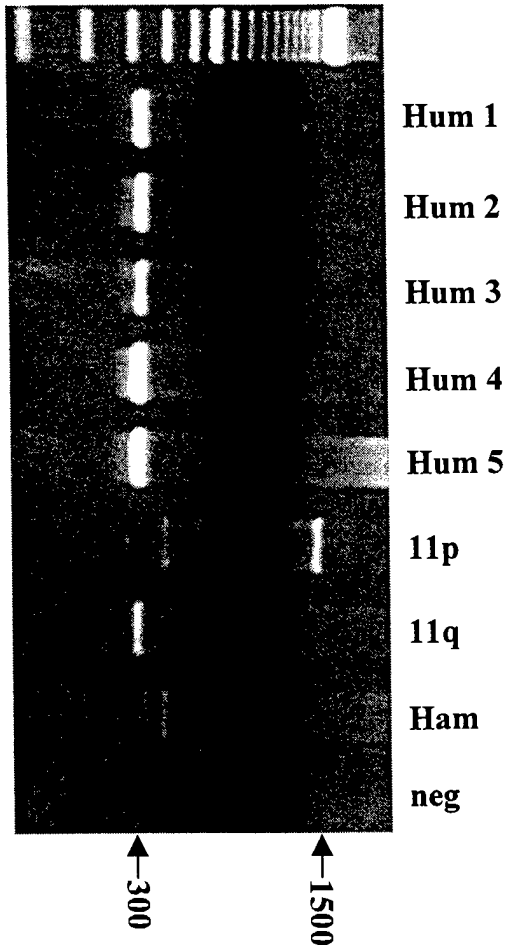
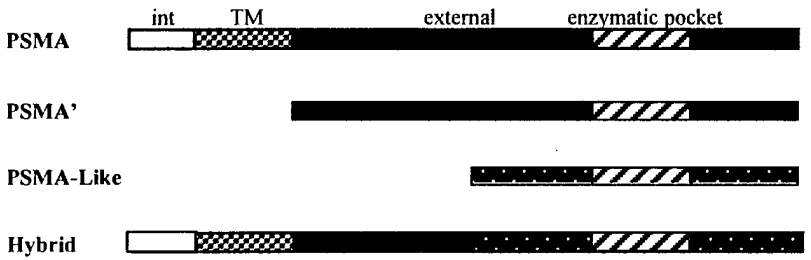
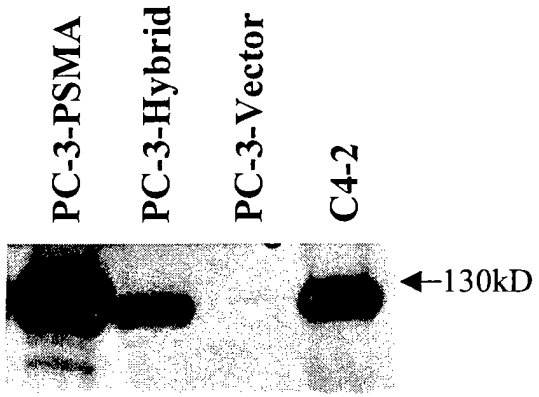


Figure 8

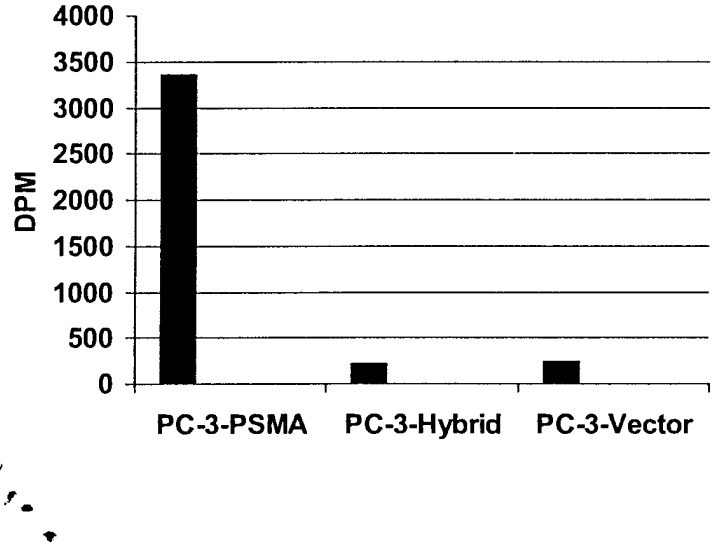
a)


int
TM
external
enzymatic pocket
PSMA
PSMA'
PSMA-Like
Hybrid

b)


PC-3-PSMA
PC-3-Hybrid
PC-3-Vector
C4-2
130kD

c)


4000
3500
3000
2500
2000
1500
1000
500
0
DPM
PC-3-PSMA
PC-3-Hybrid
PC-3-Vector